

Development of JNK2-Selective Peptide Inhibitors That Inhibit Breast Cancer Cell Migration

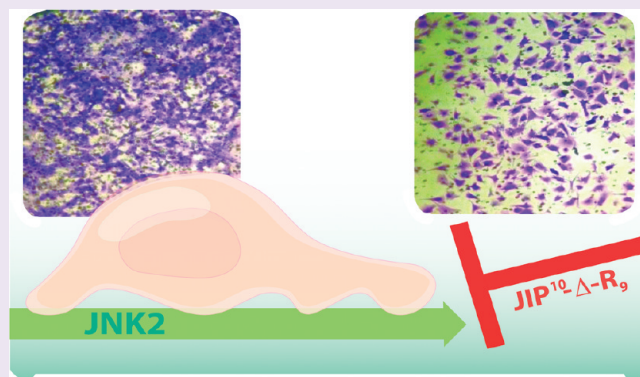
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ABSTRACT: Despite their lack of selectivity toward c-Jun N-terminal kinase (JNK) isoforms, peptides derived from the JIP (JNK Interacting Protein) scaffolds linked to the cell-penetrating peptide TAT are widely used to investigate JNK-mediated signaling events. To engineer an isoform-selective peptide inhibitor, several JIP-based peptide sequences were designed and tested. A JIP sequence connected through a flexible linker to either the N-terminus of an inverted TAT sequence (JIP¹⁰-Δ-TAT¹) or to a poly arginine sequence (JIP¹⁰-Δ-R₉) enabled the potent inhibition of JNK2 (IC₅₀ ≈ 90 nM) and exhibited 10-fold selectivity for JNK2 over JNK1 and JNK3. Examination of both peptides in HEK293 cells revealed a potent ability to inhibit the induction of both JNK activation and c-Jun phosphorylation in cells treated with anisomycin. Notably, Western blot analysis indicates that only a fraction of total JNK must be activated to elicit robust c-Jun phosphorylation. To examine the potential of each peptide to selectively modulate JNK2 signaling *in vivo*, their ability to inhibit the migration of Polyoma Middle-T Antigen Mammary Tumor (PyVMT) cells was assessed. PyVMTjnk2^{-/-} cells exhibit a lower migration potential compared to PyVMTjnk2^{+/+} cells, and this migration potential is restored through the overexpression of GFP-JNK2α. Both JIP¹⁰-Δ-TAT¹ and JIP¹⁰-Δ-R₉ inhibit the migration of PyVMTjnk2^{+/+} cells and PyVMTjnk2^{-/-} cells expressing GFP-JNK2α. However, neither peptide inhibits the migration of PyVMTjnk2^{-/-} cells. A control form of JIP¹⁰-Δ-TAT¹ containing a single leucine to arginine mutation lacks ability to inhibit JNK2 *in vitro* cell-free and cell-based assays and does not inhibit the migration of PyVMTjnk2^{+/+} cells. Together, these data suggest that JIP¹⁰-Δ-TAT¹ and JIP¹⁰-Δ-R₉ inhibit the migration of PyVMT cells through the selective inhibition of JNK2. Finally, the mechanism of inhibition of a D-retro-inverso JIP peptide, previously reported to inhibit JNK, was examined and found to inhibit p38MAPKα in an *in vitro* cell-free assay with little propensity to inhibit JNK isoforms.



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JNKs (c-Jun N-terminal kinases) are members of the mitogen-activated protein kinase (MAPK) family, which regulate various cellular functions.¹ In response to stress, they can convey pro-inflammatory, mitogenic, or apoptotic signals.² In mammals JNKs are encoded by three different genes (*jnk1*, *jnk2*, and *jnk3*), which may alternatively splice to produce a total of 10 different isoforms. The *jnk1* and *jnk2* genes are expressed ubiquitously, whereas *jnk3* is expressed mainly in testes and neuronal tissues, including the brain with low levels also found in cardiac myocytes.³

Jnk genes are implicated in several diseases such as type I and type II diabetes, Alzheimer's disease, arthritis, asthma, atherogenesis, heart failure, and Parkinson's disease.⁴ Because of its important role in regulating both apoptosis and proliferation, JNK can act as a tumor promoter and a tumor suppressor in cancer,^{5,6} both in a tissue- and stimulus-specific manner. Overall, from the perspective of tumor biology, JNK mediates the transforming actions of oncogenes such as Ras and Bcr-Abl.⁷ A causal relationship between JNK activation and

accelerated tumor growth has been reported in several studies,⁸ as the antisense JNK oligonucleotides were found to inhibit the growth of tumor cells (PC12, A549, HeLa, and MCF-7).⁹

Compared to *jnk1* and *jnk3*, the *jnk2* gene has a dominant role in cancer. For example, JNK2 is implicated in tumorigenesis *via* activation of Akt and overexpression of eukaryotic translation initiation factor 4 (eIF4E) in a human glioblastoma model.¹⁰ JNK2 is also constitutively activated in glial tumor cell lines, further supporting its tumorigenic role.¹¹ Moreover, JNK2-knockout mice displayed lower growth of chemically induced papillomas compared to wild type.¹²

The Van Den Berg laboratory recently reported that JNK2 knockout mice expressing the Polyoma Middle T Antigen transgene

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Table 1. Selectivity of JIP Peptides

Peptide	Sequence	JNK1 IC ₅₀ (μM) ^a	JNK2 IC ₅₀ (μM) ^a	JNK3 IC ₅₀ (μM) ^a	p38MAPKα IC ₅₀ (μM) ^a	ERK2 IC ₅₀ (μM) ^a
JIP ¹⁰	Ac-PKRPTTLNLF-amide 10 mer JIP	1±0.05	2±0.15	2.6±0.07	168±4	180±7
TAT-JIP ¹⁰	Ac-YGRKKRRQRRR-RPKRPTTLNLF-amide TAT-11 mer JIP	1.1±0.1	1.8±0.2	1.9±0.18	–	–
TAT- <i>ppp</i> -JIP ²⁰	Ac-GRKKRRQRRR-PP-RPKRPTTLNLFQVPRSQDT-amide TAT- <i>bip</i> roline linker-20 mer JIP	0.33±0.01	0.45±0.04	0.06±0.005	1.45±0.1	179±18
JIP ¹⁰ -Δ-TAT ⁱ	Ac-PKRPTTLNLF-AHX-RRRQRRKKRG-amide ^b 10 mer JIP-AHX Linker-Inverse TAT	1.16±0.05	0.092±0.003	1.2±0.07	13.7±2.9	161±8
JIP ¹⁰ -Δ-R ₉	Ac-PKRPTTLNLF-AHX-RRRRRRRRR-amide 10 mer JIP-AHX Linker-Poly Arginine	1.1±0.13	0.089±0.005	1.2±0.09	14.1±1.4	206±5.8
<i>m</i> JIP ¹⁰ -Δ-TAT ⁱ	Ac-PKRPTTLNLF-AHX-RRRQRRKKRG-amide 10 mer JIP Arg mutant-AHX Linker-Inverse TAT	1900±37	420±30	900±81	447±14	580±30

^a IC₅₀ determined as described in Methods. ^b Δ: aminohexanoyl.

developed mammary tumors showed higher tumor multiplicity but lower proliferation rates.¹³ Cell lines derived from these tumors provided useful tools to evaluate the potential function of JNK2 in various breast cancer phenotypes including cell migration.¹⁴

Cell migration contributes to tissue repair and regeneration, mental retardation, atherosclerosis, arthritis, and embryonic morphogenesis,¹⁵ and migration is critically important in driving cancer metastasis. Mitogen-activated protein kinases, including JNK, p38-MAPKα, and extracellular-signal-regulated protein kinase (ERK) play crucial roles in promoting cell migration.¹⁶ Biochemically, several JNK substrates such as IRS-1, p66Shc, and paxillin promote cell migration.⁷ In fact, *jnk1* was implicated in embryonic epithelial cell migration by Weston *et al.*, who showed a delay in eyelid closure resulting from corneal epithelial cell migration, in *jnk1*−/−;*jnk2*+ /− mice compared to their *jnk1*+ /+;*jnk2*+ /− littermates.¹⁷

Recent literature underscores the importance of JNKs as attractive targets for treatment of a variety of diseases, which has triggered extensive drug discovery efforts. In our investigation, our goal was to identify a JNK-specific inhibitor and, ideally, a JNK2 isoform-selective inhibitor that acts therapeutically to treat various JNK2-associated diseases, including cancer.

Due to the specificity limitations of most JNK inhibitors designed to bind in the ATP binding site, several groups have focused on identifying small molecule¹⁸ or peptide inhibitors that bind to JIP-JNK interaction sites. For example, recently, the small molecule BI-78D3, which has an IC₅₀ of 500 nM was reported.¹⁹ The JIP (JNK Interacting Protein) scaffolds, including JIP1, JIP2, JIP3, and JIP4, bind to both JNK and MKK7 and potentiate JNK activation. JIP1 is expressed in many tissue types, including neuronal, neuroendocrine, pulmonary, and renal, among others.²⁰ JIP-based inhibitors have been developed through the use of the single D-domain (D-site) of JIP1, consisting of 11 amino acids (153–163) that correspond to the JIP1 docking site of JNK. This 11-mer peptide (pepJIP1) acts as a specific inhibitor of JNK, which binds to inactive JNK1, as elucidated by crystallization, and functions through an allosteric inhibition mechanism.²¹ PepJIP1 inhibits JNK activity in *in vitro* cell-free assays toward recombinant c-Jun, Elk, and ATF2 and displays remarkable selectivity for the JNKs with little inhibition of the closely related MAPKs ERK and p38MAPKα.²¹

While JNK1 inhibition by pepJIP1 occurs mainly through direct competition with a docking site (the D-site) of substrates or upstream kinases, allosteric effects may contribute to its potency and specificity. To increase cell permeability, pepJIP1 was fused with the HIV-TAT (Human Immuno-deficiency Virus-Trans-acting activator of transcription) peptide. Its administration in both genetically and diet-induced mouse models of insulin resistance and Type 2 diabetes restores normoglycemia without causing hypoglycemia in lean

mice.²² However, uncertainties remain regarding the specificity and selectivity toward different JNK isoforms for this group of peptides. Such characteristics potentially significantly affect their use as efficient therapeutic agents in targeting definitive active sites in the human body.

In this work, we investigated the role of JNK2 in cell migration and developed a JNK2-selective inhibitor that efficiently inhibits cell migration in a breast cancer cell line.

RESULTS AND DISCUSSION

Metastasis, the therapeutically elusive trait of cancer cells, is often regarded as an early event in tumor progression. It initiates through the up-regulation of motility genes, priming a specific pool of tumor cells to develop a migratory phenotype. These cells gain an adaptive advantage to respond to microenvironmental cues necessary to initiate proteolytic invasion of the surrounding matrix. Therefore, proteins involved in cancer cell migration are potential targets of antimetastatic therapy. In this manuscript we describe two peptides with 10-fold selectivity for JNK2 over JNK1 and JNK3, which inhibit JNK docking interactions. These cell-permeable peptides inhibit the ability of cell lines derived from tumors arising from Polyoma Middle T Antigen (PyVMT) to undergo cell migration and support the notion that JNK2-selective inhibitors may be useful therapeutic targeting molecules against breast cancer metastasis.

Examining the Specificity of JIP-Based Peptide Inhibitors. The ability of JIP peptide inhibitors to selectively bind JNK1, 2, and 3 versus other MAPKs is well documented.^{21,23,24} However, an isoform-specific JIP peptide inhibitor has not been reported. To explore the specificity of the JIP peptides a 10-mer JIP_{144–153} peptide (JIP¹⁰: PKRPTTLNLF) was synthesized and tested in *in vitro* kinase assays. This peptide is composed of the amino acids 144–153 of the JNK-binding domain of the JIP-1 scaffold protein. When tested in kinase assays, JIP¹⁰ inhibited the JNKs (Figure 1a) but did not show any selectivity toward the three JNK isoforms (Table 1), which were inhibited with IC₅₀'s of 1.0–2.6 μM. As expected JIP¹⁰ exhibited a minimal ability to inhibit ERK2 (IC₅₀ = 180 μM) and p38MAPKα (IC₅₀ 168 μM). We reasoned that the absence of JNK isoform selectivity reflects the highly conserved nature of the D-recruiting site (DRS) of the JNKs, which binds the JIP¹⁰ sequence.²⁵

Cell-penetrating peptides (CPP) have been used widely to deliver impermeable molecules into intact cells. A peptide from the TAT-HIV protein is one of the most frequently used CPPs.²⁶ Thus, the arginine-rich domain of TAT, consisting of the amino acids 49–58 (TAT: GRKKRRQRRR), was employed to develop cell-permeating JIP peptides. Using a cell-penetrating peptide

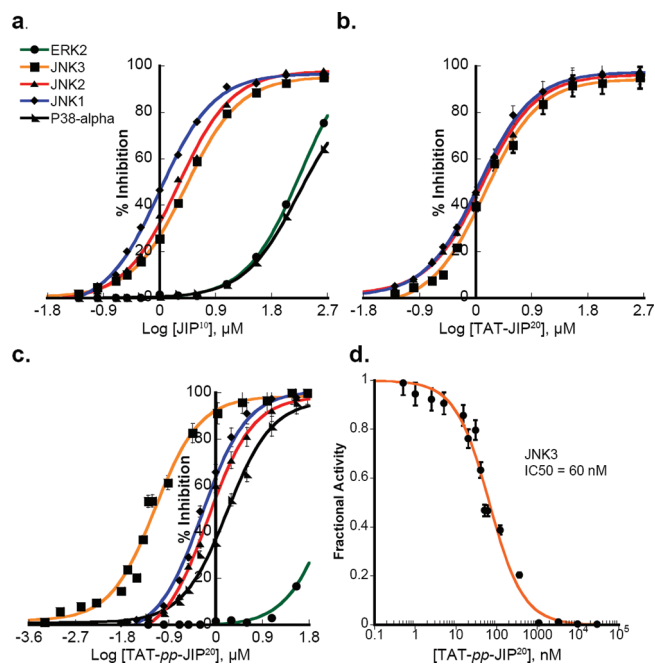


Figure 1. Selectivity of previously reported JIP peptides. The effect of peptides on the ability of JNKs to phosphorylate GST-c-JUN (1–221) and p38MAPK α or ERK2 to phosphorylate a peptide substrate was tested. (a) JIP¹⁰ is non-selective toward specific JNK isoforms. (b) The cell-permeable peptide TAT-JIP¹⁰ does not show selectivity toward any of the three JNK isoforms. (c) The cell-permeable peptide TAT-pp-JIP²⁰ exhibits 5-fold greater selectivity for JNK3 over JNK1 and JNK2 but inhibits p38MAPK α , JNK1, and JNK2 with similar potency. (d) TAT-pp-JIP²⁰ exhibits marked potency toward JNK3, with an IC₅₀ of \sim 60 nM, as determined by fitting the fractional activity at every inhibitor concentration to Morrison's equation for a tight-binding inhibitor.³⁵

consisting of the TAT_{48–58} sequence connected directly to the N-terminus of an 11-mer JIP_{143–153} peptide (TAT-JIP¹⁰: YGRKKRRQRRR-RPKRPTTLNLF), Zhang *et al.* demonstrated inhibition of JNK3 activation *in vivo*, resulting in neuronal protection against ischemic brain injury.²⁷ When we tested this peptide in *in vitro* kinase assays, we found it displayed no isoform selectivity (Figure 1b), inhibiting JNK1, 2, and 3 with IC₅₀ values of 1.1–1.9 μ M (Table 1). Thus, compared to JIP¹⁰ the N-terminal TAT sequence of TAT-JIP¹¹ provides no additional selectivity among the JNK isoforms.

Bonny *et al.* (2001) engineered a longer cell-permeating peptide inhibitor of JNK by connecting the HIV-TAT_{49–58} cell-penetrating sequence to the N-terminus of the 20 amino acid inhibitory domain of JIP-1 (143–162). The two are joined by two proline residues, which act as a flexible linker between the TAT and the JIP sequences.²⁸ We synthesized this peptide and tested its potency toward different MAP kinases in *in vitro* kinase assays. Interestingly, this peptide (TAT-pp-JIP²⁰: GRKKRRQ-RRR-PP-RPKRPTTLNLFQVPRSQDT) exhibited a 5–7-fold selectivity for JNK3 over JNK1 and JNK2 with an IC₅₀ of 60 nM for JNK3 (Figure 1d). However, we found that it inhibits p38MAPK α , JNK1, and JNK2 with similar potency (Figure 1c), with IC₅₀'s of 1.45 ± 0.1 , 0.33 ± 0.01 , and 0.5 ± 0.05 μ M, respectively (Table 1).

Bonny *et al.* (2001) also developed a cell-stable JIP-peptide composed of the D-retro-inverso form of TAT-pp-JIP²⁰ (D-TAT-pp-JIP²⁰).²⁸ This peptide was proposed to have a beneficial

effect on several neuronal disorders including cerebral ischemia,²⁹ stroke, and Parkinson's disease³⁰ and was recently classified as a possible novel therapy for diabetes.²² All such effects were attributed to its inhibition of the JNK pathway in the cell, but the precise mechanism of neuroprotection remains unclear.³¹ Therefore the specificity of D-TAT-pp-JIP²⁰ toward the three MAPK families was examined using the kinase assay described above. In addition, the isoform specificity of two *retro-inverso* peptides corresponding to residues 143–152 (D-JIP¹⁰) and 143–162 (D-JIP²⁰) were examined. D-JIP¹⁰ and D-JIP²⁰ showed negligible inhibition toward the five MAP kinases tested (Table 2). Recently we studied the specificity of a docking interaction between JNK1 and the scaffolding protein JIP1.²⁵ Free energies for the binding of JIP¹⁰ and D-JIP¹⁰ were estimated using molecular mechanics Poisson–Boltzmann and Generalized-Born surface area (MM-PB/GBSA) methods. The binding free energy calculations predicted that the binding of the JIP (D) form (D-JIP¹⁰) to JNK1 is significantly weaker than the binding of the JIP (L) form (JIP¹⁰). This was also confirmed experimentally.²⁵

Interestingly, D-TAT-pp-JIP²⁰ exhibited a moderate ability to inhibit JNK3 (IC₅₀ \approx 76 μ M), with a potency 4–5 times higher than that of JNK1 and JNK2 (IC₅₀ 290 \pm 25 and 350 \pm 80 μ M, respectively) (Figure 2a). Surprisingly, it exhibits even greater potency toward p38MAPK α (Figure 2a). Thus, D-TAT-pp-JIP²⁰ fully inhibits the phosphorylation of a peptide substrate by p38MAPK α with an IC₅₀ of 1.8 ± 0.18 μ M and can inhibit the phosphorylation of GST-ATF2 (Δ 1–115) by p38MAPK α with an IC₅₀ of 14 ± 0.1 μ M (Figure 2b). These data suggest that p38MAPK α (and not the JNKs) is a potential target of D-TAT-pp-JIP²⁰ *in vivo*.

Taken together, the studies described above suggest that the extension of peptides beyond the DRS docking domain can lead to changes in selectivity and potency. Therefore, we decided to engineer a new generation of cell-penetrating JIP-based peptides. In doing so, we changed the position of the TAT peptide relative to the JIP sequence and introduced a flexible linker that connects the JIP and CP domains together. After testing several designed sequences, we developed a peptide with significant selectivity for JNK2. The backbone of this peptide consists of the 10-mer JIP_{144–153} peptide connected to the N-terminus of an inverted HIV-TAT_{49–58} sequence through a flexible six-carbon linker formed by a 6-amino-hexanoic acid group (JIP¹⁰- Δ -TATⁱ: PKRPTTLNLF- Δ -RRRQRKKRG). JIP¹⁰- Δ -TATⁱ inhibits JNK2, with an IC₅₀ of 92 nM (Figure 3a) exhibiting a 10- to 12-fold selectivity over JNK1 (IC₅₀ = 1.2 ± 0.05 μ M) and JNK3 (IC₅₀ of 1.2 ± 0.07 μ M) (Table 1). Moreover, JIP¹⁰- Δ -TATⁱ did not exhibit marked potency toward p38MAPK α or ERK2 (Figure 3b). Similarly, we developed another peptide with the same backbone as JIP¹⁰- Δ -TATⁱ but instead used nine arginine residues in place of the TAT sequence to enhance the cell penetration (JIP¹⁰- Δ -R⁹: PKRPTTLNLF- Δ -RRRRRRRRR). JIP¹⁰- Δ -R⁹ exhibited a similar selectivity to that of JIP¹⁰- Δ -TATⁱ, with high potency toward JNK2 (IC₅₀ = 89 nM) (Figure 3c and d).

JIP¹⁰- Δ -TATⁱ and JIP¹⁰- Δ -R⁹ Are Efficient Cell-Penetrating Peptides. Before testing the *in vivo* bioactivity of the developed peptides, we investigated whether these peptides can be translocated efficiently into breast cancer cells. To do so, each peptide was labeled at its C-terminus on a Cys residue with FITC (fluorescein isothiocyanate dye). *In vivo* localization of JIP¹⁰- Δ -TATⁱ and JIP¹⁰- Δ -R⁹ was accomplished using either 4T1.2 murine mammary cancer cells treated with 2 μ M FITC-labeled JIP¹⁰- Δ -TATⁱ (FITC-JIP¹⁰- Δ -TATⁱ) or PyVMT murine mammary cancer cells treated with 2 μ M FITC-labeled peptides (FITC-JIP¹⁰- Δ -TATⁱ or FITC-JIP¹⁰- Δ -R⁹).

Table 2. Selectivity of *retro-inverso* JIP Peptides

Peptide	Sequence	JNK1 IC ₅₀ (μM) ^a	JNK2 IC ₅₀ (μM) ^a	JNK3 IC ₅₀ (μM) ^a	p38MAPKα IC ₅₀ (μM) ^a	ERK2 IC ₅₀ (μM) ^a
D-JIP ¹⁰	Ac-FLNLTTPRKP-amide Retro-inverso JIP ¹⁰	5900±307	2500±171	1800±95	1000±79	
D-JIP ²⁰	Ac-TDQSRPVQPFELNLTTPRKPR-amide Retro-inverso JIP ²⁰	2200±25	1500±109	415±28	1400±149	
D-TAT- <i>pp</i> -JIP ²⁰	Ac-TDQSRPVQPFELNLTTPRKPR-PP-RRRQRRKKRG-amide Retro-inverso of JIP ¹⁰ -Δ-TAT ⁱ	288±24	355±79	76.3±6.8	1.87±0.18	206±37

^a IC₅₀ determined as described in Methods. D: *retro-inverso*.

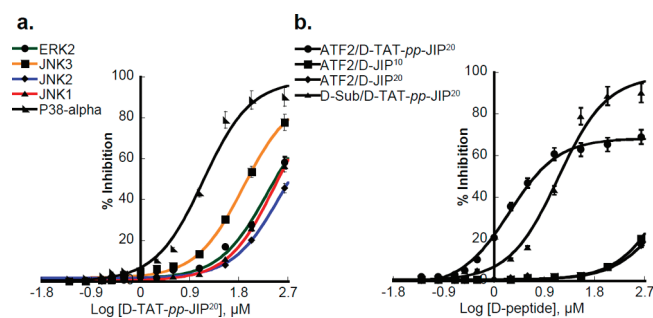


Figure 2. Selectivity of *retro-inverso* forms of JIP peptide. (a) The effect of the cell-permeable peptide D-TAT-*pp*-JIP²⁰ on the ability of JNK to phosphorylate GST-c-JUN (1–221) and of p38MAPKα and ERK2 to phosphorylate a peptide substrate was examined. D-TAT-*pp*-JIP²⁰ is a moderately more potent inhibitor of p38MAPKα than the three JNK isoforms. D-TAT-*pp*-JIP²⁰ weakly inhibits JNK3 and marginally inhibits JNK1, JNK2, and ERK2. (b) The phosphorylation of 10 μM GST-ATF2 (1–115) by p38MAPKα is not affected by D-JIP¹⁰ or D-JIP²⁰, whereas D-TAT-*pp*-JIP²⁰ diminishes the ability of p38MAPKα to phosphorylate GST-ATF2 (IC₅₀ ≈ 1.9 μM), and the peptide substrate (IC₅₀ ≈ 14 μM).

Live cell images were captured of highly metastatic 4T1.2 murine mammary carcinoma cells (Figure 4a) revealing that the FITC-JIP¹⁰-Δ-TATⁱ peptide efficiently penetrated into the cytoplasm and nucleus within 30 min of incubation. The case was similar for PyVMT cells, as both labeled peptides (FITC-JIP¹⁰-Δ-TATⁱ or FITC-JIP¹⁰-Δ-R⁹) could rapidly enter PyVMT cells once added to the culture medium. Figure 4b shows images of live PyVMT cells captured after 15 and 60 min of peptide treatment. Notably, although these peptides rapidly penetrate into the cytoplasm of PyVMT cells within minutes of addition to the culture medium, their entrance into the nucleus requires up to 1 h.

Effects of Peptides on JNK Activation and c-Jun Phosphorylation in HEK293T Cells. JIP peptides are known to work as JNK inhibitors by acting as a barrier to the interaction of JNKs with the upstream kinases MKK4 and MKK7 and downstream substrates, *e.g.*, c-Jun.²¹ Western blot analysis was employed to examine the effect of peptide treatment (5–100 μM, 16 h) on the activation of JNK or the phosphorylation of c-Jun, following stimulation of JNK by anisomycin (Figure 5). Phosphorylation of c-Jun, an *in vivo* substrate of the JNKs³¹ is strongly inhibited by both JIP¹⁰-Δ-TATⁱ and JIP¹⁰-Δ-R⁹ in cell-free *in vitro* assays (Figure 3). The Western blot analysis reveals that the incubation of HEK293T cells with either peptide leads to a dose-dependent decrease in c-Jun phosphorylation. Either peptide at a concentration of 5 μM results in more than a 50% inhibition of c-Jun phosphorylation, and 95% inhibition was achieved at higher concentrations (*ca.* 100 μM) (Figure 5). Interestingly, either peptide at 5 μM was enough to inhibit the activation/phosphorylation of cellular JNKs by ~90%, and the inhibitory potency of JIP¹⁰-Δ-R⁹ was clearly higher than that

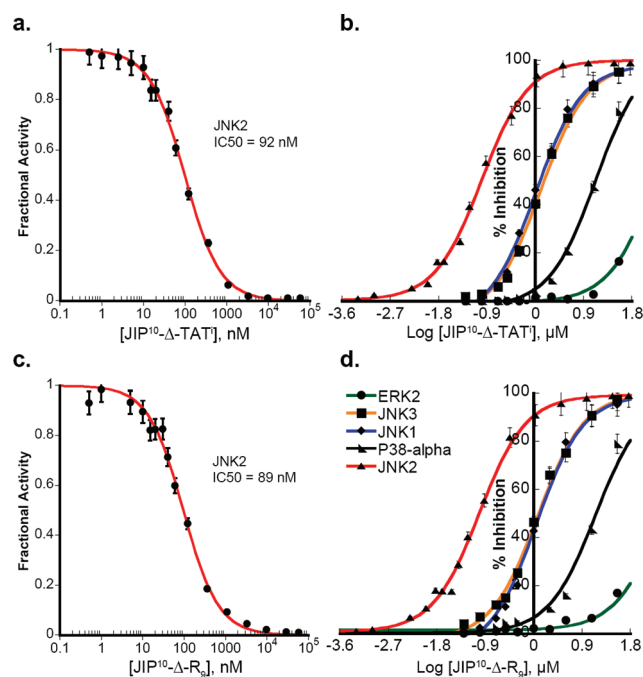


Figure 3. Selectivity of newly engineered peptide inhibitors toward JNK2. (a) JIP¹⁰-Δ-TATⁱ is a potent and selective inhibitor toward JNK2, with an IC₅₀ of ~92 nM as determined by fitting the fractional activity at every inhibitor concentration to Morrison's equation for a tight-binding inhibitor.³⁵ (b) JIP¹⁰-Δ-TATⁱ exhibits 10-fold greater selectivity for JNK2 compared to JNK1 and JNK3, while maintaining limited potency toward p38MAPKα (150-fold less than JNK2) and ERK2. (c) JIP¹⁰-Δ-R⁹ exhibits an IC₅₀ of ~89 nM toward JNK2 (fitting to Morrison's eq³⁵ for tight-binding inhibitor). (d) JIP¹⁰-Δ-R⁹ exhibits a 10-fold higher selectivity for JNK2 over JNK1 and JNK3 with limited potency toward p38MAPKα and ERK2.

of JIP¹⁰-Δ-TATⁱ especially at concentrations ≥ 10 μM. As a control, cells were incubated with *m*JIP¹⁰-Δ-TATⁱ, which has the same sequence as JIP¹⁰-Δ-TATⁱ except for a single point mutation (L150R) in JIP_{144–153}, which renders it incapable of inhibiting any of the MAP kinases tested (Table 1). As shown in Figure 5, 100 μM JIP¹⁰-Δ-TATⁱ inhibits neither JNK activation nor c-Jun phosphorylation, strongly supporting the notion that JIP¹⁰-Δ-TATⁱ and JIP¹⁰-Δ-R⁹ directly bind JNKs and prevent their interactions with upstream and downstream protein targets. These studies demonstrate the efficacy of both peptides in a cell-based system and reveal that only a fraction of total JNK must be activated to elicit robust signaling to a downstream substrate.

Assessing JNK2-Specific Peptides in Breast Cancer Cells. To date, a cellular assay specific for endogenous JNK2 has not been developed. Recently, however the Van Den Berg laboratory revealed a specific role for JNK2 in mammary cancer cell

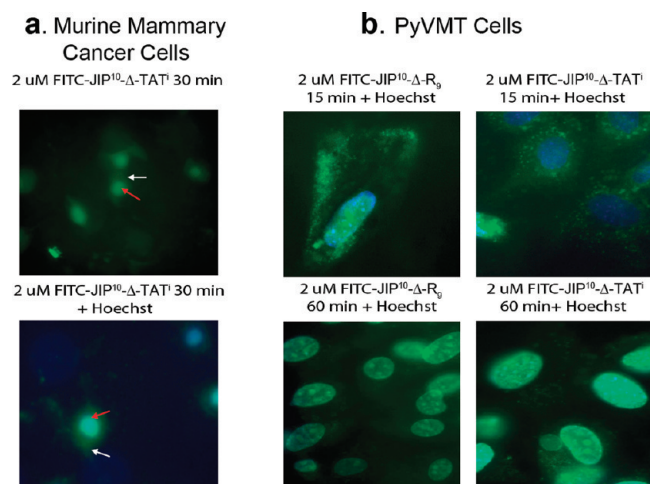


Figure 4. Cellular uptake of JIP peptides. (a) Cellular uptake of FITC labeled JIP¹⁰-Δ-TATⁱ was observed as a bright green signal (white arrow for the cytoplasm, red for the nucleus) in 4T1.2 murine mammary carcinoma cells. (b) JIP¹⁰-Δ-R₉ and JIP¹⁰-Δ-TATⁱ internalization was visualized as robust green punctuates in the cytoplasm of PyVMTjnk2+/+ cells, 15 min after addition, and the peptide was retained up to 1 h post addition. Higher magnification shows relatively higher uptake of JIP¹⁰-Δ-R₉ in the early time point. However, peptide retention was more efficient with the JIP¹⁰-Δ-TATⁱ peptide. Images are representative of data from at least 4 independent experiments.

migration using the PyVMT model of mammary tumorigenesis.¹³ The PyVMT model is important because of its close resemblance to the actual human disease both in tumor progression stages and in genetic alterations: notably, Src, Shc, and PI3K signaling are upregulated in the PyVMT model, and their signaling is also often upregulated in human breast tumors.¹³ Cell lines derived from PyVMT jnk2+/+ or jnk2-/- tumors were verified with respect to JNK2 expression by both qPCR and Western blotting.¹⁴ Furthermore, JNK1α 1/2 (p55), JNK1β2 (p46), and JNK3 were shown to be expressed similarly in both cell lines.¹⁴

We utilized a recent observation that the migration potential of PyVMTjnk2+/+ cells is reproducibly 5-fold greater than that of PyVMTjnk2-/- cells.¹⁴ This observation provided a JNK2-specific functional assay with which to test the inhibitor, since the exogenous expression of GFP-JNK2α in the PyVMTjnk2-/- cells significantly restored the ability of the cells to migrate.¹⁴

Therefore, we examined whether JIP¹⁰-Δ-TATⁱ and JIP¹⁰-Δ-R₉ inhibited directional breast cancer cell migration in these PyVMT cell lines. As a control we also utilized *m*JIP¹⁰-Δ-TATⁱ. Thus, PyVMTjnk2+/+ cells were treated with no peptide, *m*JIP¹⁰-Δ-TATⁱ (10 μM) as a negative control, JIP¹⁰-Δ-TATⁱ (10 μM), and JIP¹⁰-Δ-R₉ (10 μM) (Figure 6). Cell viability was monitored during the migration assay and found to be unaffected by the addition of the peptides (data not shown). Untreated PyVMTjnk2+/+ cells showed robust migration, similar to those treated with the mutant peptide *m*JIP¹⁰-Δ-TATⁱ. In contrast, the JIP¹⁰-Δ-TATⁱ and especially JIP¹⁰-Δ-R₉ displayed a strong inhibition of cell migration. These data are consistent with the observed potency of the peptides toward JNK2 in both cell-free (Table 1) and cell-based *in vitro* assays (Figure 5).

To further confirm the activity of the newly engineered peptides toward JNK2 *in vivo*, we used PyVMTjnk2-/- cells expressing GFP or GFP-JNK2α2 and tested their ability to

migrate when treated with *m*JIP¹⁰-Δ-TATⁱ (10 μM), JIP¹⁰-Δ-TATⁱ (10 μM), or JIP¹⁰-Δ-R₉ (10 μM). None of the peptides affected the migration of PyVMTjnk2-/- cells expressing GFP alone indicating that the absence of JNK2α in these cells renders JNK2-selective peptides ineffective with respect to the further inhibition of cell migration (Figure 7a and b).

However, the exogenous expression of GFP-JNK2α2 in PyVMTjnk2-/- cells, which restores the capacity of PyVMTjnk2-/- cells to migrate, once again sensitized them to the JNK2-selective peptides. Thus, whereas the mutant peptide *m*JIP¹⁰-Δ-TATⁱ (10 μM) had no effect on PyVMTjnk2-/-GFP-JNK2α2 cell migration, the JNK2-selective peptides JIP¹⁰-Δ-TATⁱ and JIP¹⁰-Δ-R₉ both inhibited migration (Figure 7a and b).

To summarize, recently the Van Den Berg laboratory demonstrated that knockout of JNK2 inhibits migration of murine mammary carcinoma cells. Accordingly, the cell-permeable JNK2-selective peptide inhibitors (JIP¹⁰-Δ-TATⁱ and JIP¹⁰-Δ-R₉) reduced PyVMTjnk2+/+ cell migration to a level similar to the migration of PyVMTjnk2-/- cells. Compared to previously developed peptide inhibitors, JIP¹⁰-Δ-TATⁱ and JIP¹⁰-Δ-R₉ were more potent and selective toward JNK2 in *in vitro* kinase assays. Finally, with regards to the mechanism of inhibition of previously reported *D-retro-inverso* JIP peptides, the most widely used D-TAT-*pp*-JIP²⁰ showed an unexpected level of potency toward p38MAPKα and little activity toward any JNK isoform.

METHODS

Cell Culture. Mouse mammary tumor cell lines were derived from PyVMTjnk2+/+ and PyVMTjnk2-/- tumors using a previously published protocol.¹³ PyVMT jnk2-/- cells stably expressing GFP-JNK2α2, PyVMT jnk2-/- GFP-JNK2α2, were generated following selection with puromycin. Cells were maintained in DMEM F-12 media (Cellgro, Mediatech) with 10% (v/v) FBS (Gemini Bio-Products), 10 μg mL⁻¹ insulin (Humulin RTM), 5 ng mL⁻¹ EGF (PeproTech), and antibiotics. For experiments using JIP peptides, cells were preincubated with various peptides for 30 min prior to assaying for migration. The JIP peptides were maintained in the culture media for the duration of the experiment.

HEK293T cells were maintained in DMEM (Invitrogen) supplemented with 10% (v/v) FBS-US grade (Invitrogen), 2 mM L-glutamine (Invitrogen), 100 U mL⁻¹ penicillin (Sigma), and 100 g mL⁻¹ streptomycin (Sigma). Cells were cultured in a humidified 5% CO₂ incubator at 37 °C. Cells were seeded at 400,000 cells per well in a 12-well plate and incubated for 24 h before incubation with or without peptide.³² After 16 h cells were treated with anisomycin (50–100 nM) (MP Biomedicals) for 5–10 min.

Western Blot Analysis. Cells were washed in PBS (Invitrogen), and lysates were prepared in CytoBuster Protein Extraction Reagent (EMD-Biosciences). The lysates were cleared by centrifugation, and the protein concentration was measured by Bradford analysis (Bio-Rad). Lysates containing 60 μg of total protein were fractionated on a 12% SDS polyacrylamide gel (Bio-Rad) and transferred to Hybond-P PVDF Membrane (GE Healthcare). Incubation of primary antibodies was performed overnight at 4 °C using 1:10,000 antiphospho-c-Jun (Ser-63), clone Y172 rabbit mAb (Millipore); 1:2000 antiphospho-SAPK/JNK (Thr183/Tyr185) (G9) mouse mAb (Cell Signaling Technology); 1:5000 antiactin, clone 4 mouse mAb (Millipore). Either antimouse (Cell Signaling Technology) or antirabbit (Bio-Rad) horseradish peroxidase-conjugated secondary antibodies and ECL Plus Western Blotting Reagents (GE Healthcare) were used to develop the blots.

Cell Migration. Cell migration was measured using modified Boyden/transwell chamber (BD Biosciences) with 8 μm pores and coated

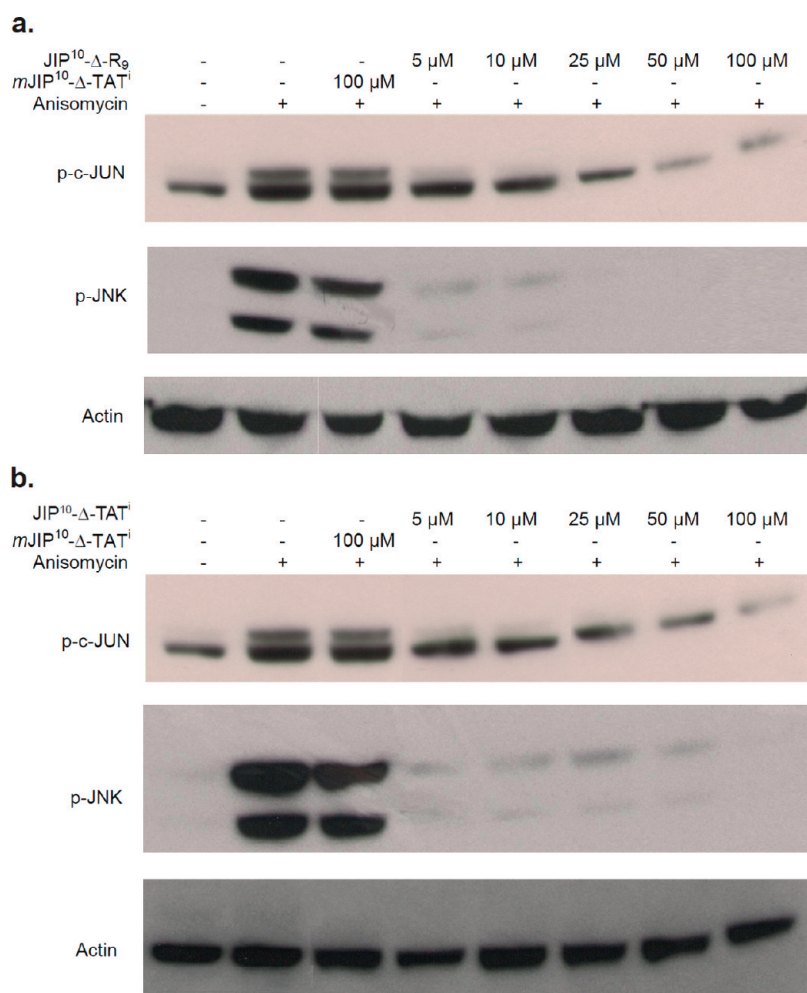


Figure 5. JIP peptides inhibit both JNK activation and c-Jun phosphorylation in HEK293 cells. (a) HEK293 cells were treated with JIP¹⁰-Δ-R₉ (5–100 μM) or *m*JIP¹⁰-Δ-TATⁱ (100 μM) for 16 h. The JNK pathway was then induced by the addition of anisomycin (50–100 nM) for 5–10 min, before lysing the cells. Lysates were fractionated by SDS PAGE (12% gel) and subjected to Western blot analysis in order to detect the phosphorylated forms of c-jun and JNK. (b) HEK293 cells were treated with JIP¹⁰-Δ-TATⁱ (5–100 μM) or *m*JIP¹⁰-Δ-TATⁱ (100 μM) and analyzed as in panel a. These results are representative of two experiments.

with 1 μg/mL fibronectin matrix. About 50,000 cells were plated on the upper chamber of the transwell insert, in serum free media (HBSS). Lower chambers were filled with HBSS (Hank's Buffered Salt Solution) medium containing 1% (v/v) FBS as the chemoattractant. Cells were allowed to migrate for 8 hrs at 37 °C. Cells were fixed in 4% paraformaldehyde and stained with 0.5% (w/v) crystal violet. Non-migrated cells in the upper chamber were removed using cotton swabs, leaving behind the cells that actively migrated to the under surface of the transwell. Migrated cells were quantified by counting four randomly chosen fields (at an objective of ×10) or by dissolving the crystal violet dye with sodium citrate and reading the absorbance (blank adjusted) at 570 nm.

JIP Internalization. About 50,000 cells were plated in 8-well chamber slides. The next day, slides were kept on ice for 10 min to stall all cellular activity and uptake. FITC-labeled JIP peptides at 10 μM were added to the medium at 37 °C and incubated for various amounts of time. The cell nucleus was briefly stained with Hoechst dye, and then the cells were imaged in real time. The fluorescent signal was detected using a CCD camera mounted on a Nikon Diaphot 300 inverted microscope.

Statistics. Results were evaluated using analysis of variance between groups, followed by nonparametric, posthoc Student's *t* test to identify differences between groups. Differences between means yielding *p* values of ≤0.05 were considered statistically significant.

MAP Kinase Activity Assay. MAP kinase assays were conducted at 30 °C in kinase assay buffer (25 mM HEPES buffer-pH 7.5, 50 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 2 mM DTT and 10 μg mL⁻¹ BSA), containing 500 μM [γ -³²P] ATP (100–1000 cpm pmol⁻¹), 11 mM MgCl₂ and different concentrations of the JIP peptides in a final total volume of 70 μL. For JNK assays, 20 nM active JNK1α1, JNK2α2, or JNK3α2 was assayed with 2 μM GST-c-jun (1–221) as protein substrate. For p38MAPKα assays, 10 nM p38MAPKα was assayed with either 10 μM D-site substrate peptide (FQRKTLQRRLKGLNLL-AHX-TGPLSPGPF, where AHX is a linker), which has a specific D-site to the MAP kinases (described in Lee *et al.*, in preparation) or 10 μM GST-ATF2 (1–115) protein substrate. For ERK2 assays 2 nM ERK2 was assayed with 10 μM D-site substrate peptide. Activity was assessed at different peptide concentrations by the measurement of initial rates. Rates were measured under conditions where the total product formation represented less than 10% of the initial substrate concentrations. The reaction was initiated by the addition of ATP. Aliquots (10 μL) were taken from every reaction at set time points (0.5, 1, 1.5, 2, 4 min) and spotted to 2 × 2 cm² squares of P81 cellulose paper. The papers were washed for 3 × 15 min in 50 mM phosphoric acid (H₃PO₄), followed by a pure acetone wash, and then dried. The amount of labeled protein was determined by counting the associated cpm on a Packard 1500 scintillation counter at a sigma value of 2.

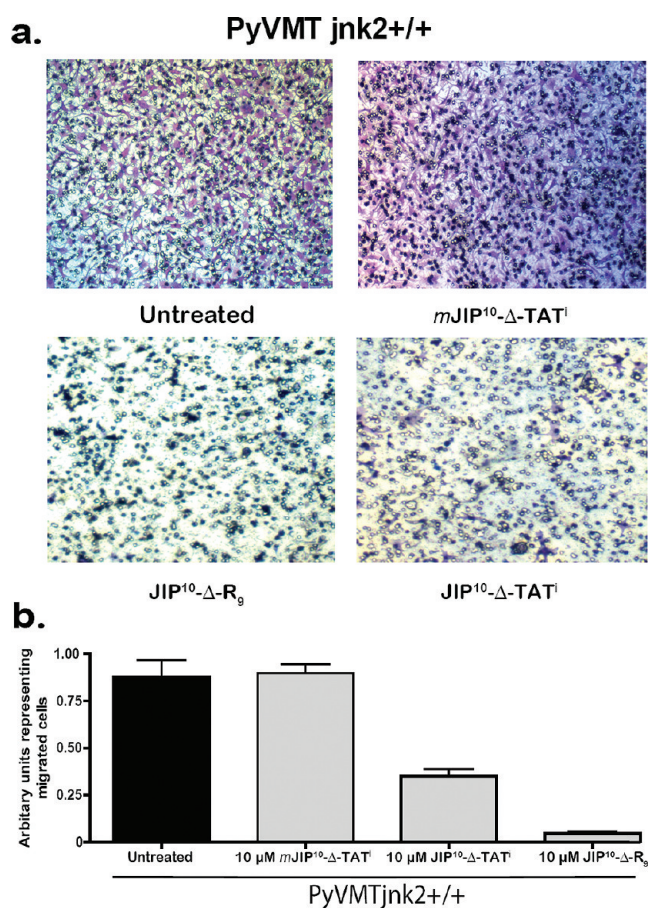


Figure 6. JIP peptides can efficiently inhibit mammary cancer cell migration. (a) Representative images of migrated cells on the underside of a transwell membrane stained with crystal violet dye. Untreated PyVMTjnk2+/+ cells migrate similar to cells treated with 10 μM $mJIP^{10}\text{-}\Delta\text{-TAT}^i$. Ten micromolar $JIP^{10}\text{-}\Delta\text{-R}_9$ and $JIP^{10}\text{-}\Delta\text{-TAT}^i$ significantly reduce the number of cells that travel through the transwell pores to successfully reach the underside of the transwell membrane (migrated cells). (b) Graphical analysis. Representative of three or more independent experiments. When compared to untreated cells a two-sided Student's *t* test analysis gave values of $p = 0.84$, 0.006, and 0.0008 for cells treated with $mJIP^{10}\text{-}\Delta\text{-TAT}^i$, $JIP^{10}\text{-}\Delta\text{-TAT}^i$, and $JIP^{10}\text{-}\Delta\text{-R}_9$, respectively.

Peptide Synthesis and Purification. D- and L-peptides were synthesized and purified using the same method described by Yan *et al.*²⁵ Peptides were synthesized on rink resin (NovaSyn TGR resin) using a peptide synthesizer (Quartet, Rainin) utilizing an Fmoc solid-phase peptide synthesis protocol. The optical rotation of each Fmoc-D-amino acid was examined to confirm their chirality by utilizing a polarimeter (241MC Polarimeter, Perkin-Elmer).

Expression, Purification, and Activation of Tagless MAPKs. Tagless full length human JNK1 α 1 (GenBank accession number NM_002750), human JNK2 α 2 (GenBank accession number NM_002752), or N-terminal truncated human JNK3 α 2 (amino acids 39–422 with alanine inserted between amino acids 39 and 40, GenBank accession number NM_138982) were expressed and purified as described previously.^{25,33} Activated tagless ERK2 (*Rattus norvegicus* mitogen activated protein kinase 1, GenBank accession number NM_053842) was expressed and purified as described elsewhere (Kaoud *et al. manuscript submitted*). DNA sequences encoding *Mus musculus* mitogen-activated protein kinase 14-p38MAPK α (GenBank accession number NM_011951) was cloned

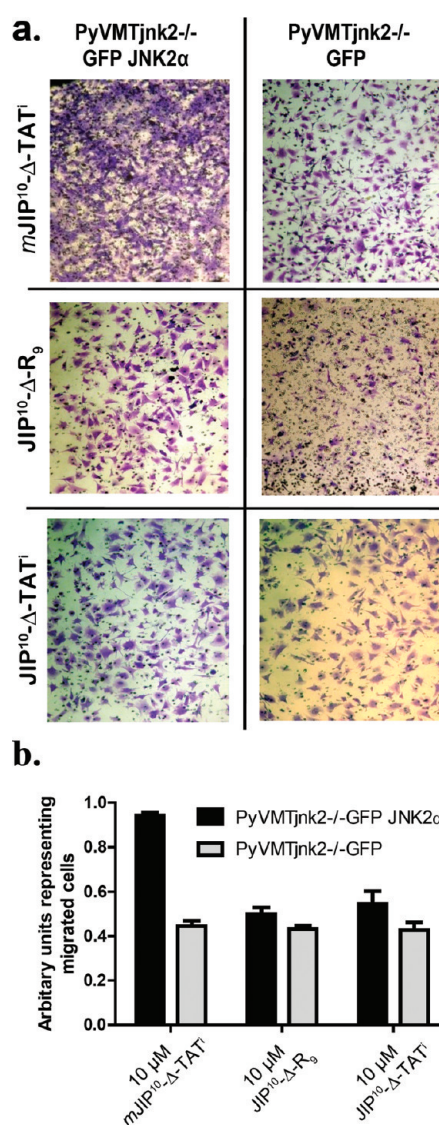


Figure 7. $JIP^{10}\text{-}\Delta\text{-R}_9$ and $JIP^{10}\text{-}\Delta\text{-TAT}^i$ specifically target JNK2-mediated cell migration. (a) Cell migration assays were performed with various JIP treatments using the isogenic PyVMTjnk2-/-GFP and PyVMTjnk2-/-GFPJNK2 cell lines. Representative fields show migrating cells with normal morphology and cellular extensions. (b) Quantification of transwell chamber-based migration assays with $mJIP^{10}\text{-}\Delta\text{-TAT}^i$, $JIP^{10}\text{-}\Delta\text{-TAT}^i$, or $JIP^{10}\text{-}\Delta\text{-R}_9$ treatment, performed in triplicate using the isogenic PyVMTjnk2-/-GFP and PyVMTjnk2-/-GFPJNK2 lines. Data is representative of at least three independent experiments. Analysis by two-sided Student's *t* test comparing the two cell lines gave values of $p = 0.0001$, 0.13, 0.15, when comparing treatments by peptides $mJIP^{10}\text{-}\Delta\text{-TAT}^i$, $JIP^{10}\text{-}\Delta\text{-R}_9$, and $JIP^{10}\text{-}\Delta\text{-TAT}^i$, respectively.

into the pET14B vector to express an N-terminal, His tagged p38MAPK α in *E. coli* BL21 (DE3) pLysS cells. The enzyme was expressed and purified as described previously.³⁴ The activated MAPKs were all stored in buffer S [25 mM HEPES (pH 7.5), 50 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 2 mM DTT and 10% (v/v) glycerol] at -80°C .

Expression and Purification of GST-c-Jun (1–221) and GST-ATF2 (1–115). GST-c-Jun (1–221) was expressed and purified according to the method of Yan *et al.*²⁵ GST-ATF2 (1–115) was expressed and purified according to the method of Szafranska *et al.*³⁴

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